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(71) Applicant: NEOGENIX, INC. [US/US]; Suite 310, 240 Cedar Knolls Road, Cedar Knolls, NJ 07927 (US).

(72) Inventors: LANSBURY, Peter, T., Jr.; Apartment 3B, 15 Davies Avenue, Brookline, MA 02146 (US). HAUSCHKA, Peter, V.; 105 Walker Lane, Needham, MA 02192 (US).

(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

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(54) Title: PURIFIED NATURAL AND SYNTHETIC COMPOUNDS FOR THE TREATMENT OF OSTEOARTHRITIS

(57) Abstract

The present invention relates to individual, well-defined compounds and the uses of these compounds, alone or in conjunction with bioactive molecules such as growth factors or metalloproteinase inhibitors, for the repair of cartilage damage as, for example, is found in osteoarthritis. Such well-defined compounds may include, but are not limited to, purified components of the extracellular matrix, derivatives of extracellular matrix components, and glycosaminoglycan mimics.

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PURIFIED NATURAL AND SYNTHETIC COMPOUNDS FOR THE TREATMENT OF OSTEOARTHRITIS

1. INTRODUCTION

The present invention relates to individual, well-defined compounds and the uses of these compounds, alone and in conjunction with bioactive molecules such as growth factors or metalloproteinase inhibitors, for the repair of cartilage damage as, for example, is found in osteoarthritis. Such well-defined compounds may include, but are not limited to, purified components of the extracellular matrix (ECM), derivatives of these ECM components, and glycosaminoglycan (GAG) mimics. Further, the present invention provides methods for the selection of those compounds which have an increased capacity for cartilage repair.

2. BACKGROUND OF THE INVENTION

2.1 CARTILAGE

Cartilage is a hard connective tissue whose unique material properties of durability, stiffness, resiliency, and viscoelasticity make possible the normal function of the musculoskeletal, auditory and respiratory systems. In the skeletal system, for example, essential structural components are comprised of cartilage, including synovial joints, which consist of articular hyaline cartilage, and intervertebral disks, and parts of the insertions of tendons and ligaments into bone, all of which consist of fibrous cartilage. Cartilage lacks nerves, and lymph and blood vessels, but cells within the tissue, however, are metabolically active, responding to hormonal changes, nutrient availability, oxygen tension, and mechanical loads. Although these cells, the chondrocytes, rarely divide in adults, they remain

m tabolically active in order to synthesize m lecules (mainly proteoglycans) to compensate for degradation of molecules within the matrix. A failure to replenish lost matrix molecules leads to matrix degradation, and thus cartilage impairment.

Cartilaginous tissue consists of three components: chondrocytes, extracellular water, and an abundant ECM. The tissue is formed as groups of chondrocytes gather and secrete a cartilagineous ECM which surrounds the cells. In most mature cartilage, chondrocytes contribute only about 5% of the total tissue volume, while the ECM makes up the remaining 95%. Tissue fluid, however, comprises the largest component of cartilage; depending on the type and age of the cartilage, extracellular water can contribute up to 80% of the tissue's wet weight. In addition to these components, several factors have been identified which affect cartilage formation. These factors include cartilage derived growth factor, which stimulates chondrocytes in vitro (Davidson, J.M. et al., 1985, J. Cell Biol. 100:1219-1227), the growth factor TGF-β, which stimulates the proliferation of many cell-types, including chondrocytes (Sporn, M.B. et al., 1986, Science 233:532-534), connective tissue activating peptides and insulin-like growth factor (IGF-1, somatomedin C), which favorably influence osteoblastic and chondrocytic repair responses in vitro (Zezulak, K.M. and Green, H., 1986, Science 233:551-553), and increase proteoglycan and collagen synthesis in cartilage tissue in vivo (Daughaday, W.H. et al., 1975, Nature 235:107).

The major component of the cartilaginous ECM are collagen fibers, with type II collagen being the most abundant subtype present in hyaline cartilage, and type I collagen being most abundant in fibr us

cartilage. (Miller, E.J., 1976, Mol. Cell. Biochem. 13:165-191). In addition to collagen, proteoglycans are another major component of hyaline cartilage ECM. (Heingard, D., 1977, J. Biol. Chem. 252:1980-1989). Proteoglycans are molecules consisting of protein bound by polysaccharide glycosaminoglycan (GAG) chains. GAG chains are comprised of repeating disaccharide units containing a derivative of either glucosamine or galactosamine. In addition, each disaccharide unit contains at least one negatively charged carboxylate or sulfate group, such that GAGs form long strings of negative charges. Cartilage GAGs include hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, and keratan sulfate. Cartilaginous proteoglycans exists in nonaggregating and aggregating monomer forms. Aggregating monomers make up the bulk (85-98%) of the proteoglycan population, and consist of protein cores to which oligosaccharides and the GAGs chondroitin sulfate and keratan sulfate are bound. The aggregating proteoglycans can exist as monomers or as groups of multiple monomers plus hyaluronic acid and small proteins known as link proteins.

The negative charges along GAG chains allow proteoglycans to bind cations and a large volume of water. Because adjacent, negatively charged GAG chains repel each other, proteoglycan molecules tend to remain in an extended form, and the water is drawn inside, creating swelling pressure within the matrix. The collagen network limits proteoglycan swelling, but loss or damage to the network allows cartilage to swell, resulting in an increased water concentration and a decreased proteoglycan concentration, ultimately leading to cartilaginous tissue damage.

2.2 OSTEOARTHRITIS

Osteoarthritis (OA) is a slowly progressive degenerative joint disease which is characterized by loss of cartilage ECM proteoglycans, fibrillation of the cartilage surface, and eventual loss of collagenous matrix to expose underlying bone (Lawrence, J.S. et al., 1966, Ann. Rheum. Dis. 25:1-24). OA, the most prevalent rheumatic disorder of the musculoskeletal system, affects over 40 million Americans and about 15% of the world's population, making it one of the most common chronic disorders (Kellgren, J.H. et al., 1958, Ann Rheum. Dis. 17:388-397; Kellgren, J.H. et al., 1961, Brit. Med. J. 2:1-6; Lawrence, J.S. et al., 1966, Ann. Rheum. Dis. 25:1-24; Gordon, T., 1968, in Bennett, P.H. and Wood, P.H.N., eds., Population Studies of the Rheumatic Diseases, Excerpta Medical Foundation, New York, pp. 391-397; Mankin, H.J. et al., 1986, J. Rheumatol. 13:1130-1160). Prevalence of the disease increases from 4 per 100 in the 18-24 year age group to 85 per 100 in the 75 to 79 year age group (Moskowitz, R.D. and Brandt, K.D., 1989, in Arthritis and Allied Conditions, 11th ed., pp. 1605-1641, Lea and Febiger). The etiopathogenesis of OA has been difficult to determine, but is probably related to mechanical wear, failure of chondrocytes to maintain a balance of ECM synthesis and degradation, and also to extracartilagenous factors such as subchondral bony remodeling and synovium-mediated inflammatory events (Howell, D.S., 1986, Am. J. Med. 80: (Suppl. 4B) 24-28).

Clinically, OA is characterized by joint pain, tenderness, limitation of movement, crepitus, and inexorably progressive disability. Pathologic changes in the once smooth cartilage surfaces include

cartilage fissuring, pitting, and erosion. Erosions, initially focal, become confluent, leading to large areas of denuded surface and eventually to loss of cartilage down to the bone. The bone tissue directly under the cartilage develops cysts, becomes thickened, and the entire end of the bone exhibits hypertrophy, resulting in the loss of congruity of the cartilage surfaces. This loss leads to increased joint instability, which, in turn, leads to further stress on joint tissues, causing inflammation and seepage of joint fluid into the surrounding tissues.

A number of biochemical and metabolic alterations may be observed in the ECM surrounding the chondrocytes of osteoarthritic cartilage. For example, OA cartilage ECM contains a reduced level of proteoglycans, which play an important role in the structural integrity of cartilage (Mankin, et al., 1971, J. Bone Joint Surg. 53A:523-537; Erlich, 1985, J. Orthop. Res. 3:170-184). Also, a smaller than normal proportion of the total proteoglycan population is present in aggregates, and GAG chains are, on average, shorter than normal (Maroudas, A. et al., 1973, Ann. Rheum. Dis. 32:1-9; Mankin, H.J., 1974, N. Engl. J. Med. 291:1285-1292; Palmoski, M. et al., 1976, Clin. Chem. Acta. 70:87-95; Inerot, S. et al., 1978, Biochem. J. 169: 143-156; Mankin, H.J., et al., 1981, J. Bone Joint Surg. 63A: 131-134). In addition, although collagen concentration and collagen phenotypes are unaltered (i.e., type II collagen is predominant in normal and OA ECM), there is a loosening of the "weave" of the cartilage ECM collagen network and a reduction in the size of the collagen fibrils (Weiss, C., 1973, Fed. Proc. 32:1459-1466; Lane, J.M. et al., 1975, Arthritis Rheum. 18:553-559; Maroudas, A. et al., 1977, Ann. Rheum. Dis. 36:399-

406). Furthermore, the water content of osteoarthritic cartilage is significantly increased (Maroudas, A. et al., 1977, Ann. Rheum, Dis. 36:399-406), which may be caused by the damaged collagen fiber network, since it no longer allows collagen to restrain proteoglycan hydration and swelling.

Increased levels of proteolytic enzymes of the metalloproteinase class have been demonstrated in human and canine OA cartilage. (Martel-Pelletier, J. et al., 1984, Arthritis Rheum. 27:305-12; Pelletier, et al., 1985, Arthritis Rheum. 28:1393-1401; Pelletier, J. et al., 1987, Arthritis Rheum. 30:541-548; Dean, D.D. et al., 1989, J. Clin. Invest. 84:678-685; Altman, R.D. et al., 1989, Arthritis Rheum. 32: 1300-1307). Increased collagenase activity has also been found in OA cartilage (Pelletier, J. et al., 1983, Arthritis Rheum. 26:63-68; Erlich, M.G., 1985, J. Orthop. Res. 3:170-184). In addition, an activation in the production of tumor necrosis factor (TNF), tissue plasminogen activator (TPA), and the lymphokine interleukin-1-β is observed as OA progresses.

Cartilage also contains a molecule termed tissue inhibitor of metalloproteinases (TIMP) (Dean, D.D. and Woessner, J.F., 1984, Biochem. J. 218:277-280; Dean, D.D. et al., 1987, J. Rheumatol. 14: (Suppl.) 43-44), which inhibits metalloproteinases and collagenase. While in OA, the concentration of TIMP is either modestly increased or unchanged, metalloproteinase levels are increased 3-5 fold (Azzo, W. et al., 1986, J. Biol. Chem. 261:5434-5441; Dean, D.D. et al., 1987, J. Rheumatol. 14: (Suppl.) 43-44; Dean, D.D. et al., 1989, J. Clin. Invest. 84:678-685). The imbalance allows proteinases to escape inhibitor control, which

represents one mechanism that may lead to matrix degradation, and thus cartilage tissue failure.

2.3 TREATMENT OF OSTEOARTHRITIS

There is currently no truly effective treatment for OA. Therapeutic goals in managing OA are pain relief, increased mobility, and reduction of disability, while ultimate goals include slowing OA progression or actually bringing about its regression. Regrettably, the available therapeutic options are limited. (Arfag, A.A. and Davis, P., 1991, Drugs 41:193-201).

Nondrug treatment alternatives range from patient education concerning joint protection, to devices, such as walkers or canes, employed to correct abnormalities of the feet, to, finally, total surgical replacement of the OA joint. Drugs used for the treatment of OA symptoms include analgesics (i.e., aspirin, acetaminophen, or ibuprofen) or non-steroidal anti-inflammatory drugs (NSAIDS), which are employed to simply relieve pain and inflammation (Husby, G. et al., 1986, Clin. Rheumatol. 5:84-91; Davis, P., 1987, J. Rheumatol. 14:94-97). Although NSAIDS are one of the major groups of drugs in terms of sales and use for the management of OA among the general population (Bjelle, A. et al., 1984, J. Rheumatol. 11:493-499), their side effects have become a problem (Wilholm, B.E. et al., 1985, in Side Effects of Anti-Inflammatory Drugs, Rainsford, K.D. and Velo, G.P. eds., MTD Press Ltd., Lancaster, pp. 55-72), particularly in the elderly (Buchanan, W.W. and Kean, W.F., 1987, J. Rheumatol. 14:98-100). Virtually all NSAIDS cause gastrointestinal hemorrhage, ulceration, or perforation, while some are associated with bone marrow depression, several cause fluid retention, and

some may contribute to renal failure. These effects are particularly important because such treatments are often long-term and the reactions may be serious, even potentially fatal, especially in elderly patients. Also, the long-term application of NSAIDS or analgesics may exacerbate the progress of OA, both because of overuse of joints that are pain-free due to the drugs' properties, and, in the case of NSAIDS, by the drugs' effects on biochemical mechanisms such as cartilage and chondrocyte metabolism.

Therapeutic agents which have disease-modifying potential, rather than functioning solely to alleviate symptoms, are of great interest for the treatment of OA. The treatment aim would be to stimulate cartilage repair and to, concurrently, inhibit cartilage breakdown by suppressing the degradative effect of enzymes on cartilage while sustaining chondrocyte metabolic activity. Such agents, termed "chondroprotective," are specifically directed toward the prevention, retardation, or reversal of the OA process (Burkhardt, D. et al., 1987, Arth. Rheum. 17:3-34). Two such agents, Rumalon® (Robapharm, Ltd., Basel, Switzerland; Rejholec, V., 1987, Semin. Arth. Rheum. 17:35-53) and Arteparon® (Luitpold Werk, Munich, Germany), have been described. Rumalon® is a mixture of ECM components in a high molecular weight (10^5-$2x10^6$ D) GAG-peptide associated complex which is derived from bovine cartilage and bone marrow. Studies indicate that this complex is non-covalent and that much of its biological activity is due to its peptide components (Burkhardt, D. and Ghosh, P., 1987, Semin. Arth. Rheum. 17:3-34). Arteparon® consists of a mixture of GAG polysulfuric acid esters (molecular weight, 10^4-$2x10^4$ D) of oversulfated GAG chains containing galactosamine and hexuronic acid (Golding,

J. and Ghosh, P., 1983, Curr. Therapy Res. 34:67-80), which is derived from bovine lung and tracheal tissue.

Although studies using these two agents are encouraging for their use as chondroprotective agents (Rejholec, V., 1987, Semin. Arthritis Rheum. 17:35-53), both Rumalon® and Arteparon® are complex, poorly characterized bovine tissue extracts, and are not only, therefore, suboptimally effective, but are also unacceptable for use in the United States. The composition of Arteparon®, for example, is the subject of some controversy (Vacha, J. et al., 1984, Arzheimittelforsch 34:607-609; Nishikawa, H. et al., 1985, Arch. Biochem. Biophys. 240:146-153; Andrews, J.L. et al., 1985, Arzheimittelforsch 35:144-148). In addition, Arteparon® distribution highly favors fibrous over hyaline cartilage (Burkhardt, D. and Ghosh, P., 1986, Curr. Therapy Res. 40:1034-1053), and, further, exhibits a heparin-related anticoagulant effect which restricts its use, especially in older patients (Rejholec, V., 1987, Semin. in Arth. and Rheum. 17:35-53) Rumalon® has been especially difficult to characterize, one reason being that its individual components may act synergistically and/or in concert with each other to exert their chondroprotective effect (Burkhardt, D. and Ghosh, P., 1987, Semin. in Arth. Rheum. 17:3-34). Although Rumalon® seems to have the ability to stimulate matrix synthesis, its effect appears to be greatly dependent on conditions and tissues used in any particular study (Dean, D.D. et al., 1991, Arth. Rheum. 34:304-313), and controversy exists as to the mixture's ability to increase proteoglycan synthesis (Malemud, C.J. and Sokoloff, L., 1971, Arth. Rheum. 14:779-780).

3. SUMMARY OF THE INVENTION

The present invention relates to individual, well-defined compounds and to the uses of these compounds, alone and in conjunction with bioactive molecules, for the repair of damaged cartilage tissue of the type, for example, that is found in osteoarthritis (OA). Such well-defined compounds may include, but are not limited to, purified components of the ECM, derivatives of these components, and GAG mimics. The bioactive molecules that can be used in conjunction with these compounds may include, for example, growth factors or metalloproteinase inhibitors. Additionally, the invention describes methods for the purification and/or modification of the ECM components, and the synthesis of the GAG mimics. Further, the invention provides methods for the selection of those compounds which have an increased capacity for cartilage repair.

Unlike previous ECM-derived compounds used for the potential alleviation of OA (i.e., Rumalon® and Arteparon®), which are complex, poorly characterized extracts, the ECM components of the present invention are well-defined molecules which, therefore, provide, for the first time, a means by which to optimize both the safety and cartilage healing properties of therapeutic formulations. Such purified ECM components may include, but are not limited to, specific GAGs, such as a single species of chondroitin sulfate.

GAG mimics have never been utilized for the alleviation of OA. Such compounds, may include, but are not limited to, sulfated polysaccharides, sulfated aromatic dyes, or sulfated polysaccharide-like oligomers.

4. DETAILED DESCRIPTION OF THE INVENTION

This invention involves single, well-defined compounds and their uses, alone and in combination with other bioactive molecules, for the repair of damaged cartilage tissue as, for example, is found in OA. These well-defined compounds may include, but are not limited to, purified ECM components, derivatives of these components, and GAG mimics. Additionally, the invention provides means for the selection of compounds which have an increased capacity for cartilage repair.

Described below are methods for the purification of specific ECM components from various sources, techniques for the modification of such components and procedures for the synthesis of GAG mimics. In addition, methods are presented describing the administration of formulations containing these well-defined compounds for the repair of damaged cartilage and the treatment of OA. Further, screening techniques are described that allow for the selection of candidate cartilage repair compounds.

4.1 PURIFICATION AND MODIFICATION OF ECM COMPONENTS

The individual purified ECM components of the invention may include, but are not limited to, single, well-defined species of glycosaminoglycan (GAG) polysaccharides or proteoglycans. A single well-defined species of GAG refers, here, to a species of a narrow molecular weight range (i.e., +/- 1000 daltons), polysaccharide composition, and sulfation pattern. Likewise, a single well-defined species of proteoglycan refers to a species of a single molecular weight range (i.e., +/- 1000 daltons), core protein identity, and GAG modification pattern.

The GAGs may consist of repeating lin ar polymers of specific disaccharides. Usually one sugar of such disaccharide units is either a hexuronic acid (HexA; i.e. D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)) or a galactose monosaccharide, and the other sugar of the disaccharide unit is a hexosamine (i.e., D-glucosamine (GlcN) or D-Galactosamine (GalN). The individual purified GAG chains of the invention, therefore, may include, but are not limited to, a basic structure composed of $(HexA\text{-}GalN)_n$, $(Hex\ A\text{-}GlcN)_n$, $(Gal\text{-}GlcN)_n$, (GlcNAc-GlcNAc) or $(Gal\text{-}GalN)_n$ disaccharide units, where n is the number of disaccharide units within the GAG chain. One or both of the sugars within each disaccharide unit is sulfated at one or two positions. While the structures above connote the basic structure of the purified GAG chain, each individual chain may include, for example, differences in sulfate substitution along the chain and epimerization of specific monosaccharides within the chain (GlcA to IdoA, for example). The purified GAG chains of the invention may include, but are not limited to, individual species of chondroitin-4-sulfate, chondroitin-6-sulfate, hyaluronic acid, heparin, heparin sulfate, keratan sulfate, dermatan sulfate, or poly-N-acetyl-glucosamine, poly-N-glucosamine, and their derivatives.

The proteoglycans of the invention, which consist of core proteins to which one or more GAG polysaccharides is covalently attached, likewise consist of a well-defined, purified species. Each of these proteoglycans is of a narrow molecular weight range (i.e., +/- 1000 daltons), core protein identity, and GAG modification pattern.

The homogeneous, individual ECM components of the invention may be purified from heterogenous starting sources. The starting sources may include, but are not limited to, heterogenous populations of groups of GAG molecules which may consist, for example, of widely varying molecular weight populations of heparin sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, chitin, or chitosan. Alternatively, more complex mixtures, including, but not limited to, Arteparon® or Rumalon® may be utilized as starting sources for ECM component purification.

A general scheme by which purified GAG and/or proteoglycan ECM components may be obtained from a heterogenous mixture of molecules involves a stepwise reduction in heterogeneity until a purified species of molecules is obtained. First, the complexity of GAG species may be reduced by treatment of the mixture with agents that attack specific groups along GAG chains. Second, a further reduction of GAG complexity may be obtained by separating the components of the mixture according to molecular weight. Finally, individual species may be obtained by separating the components of any given molecular weight class by such techniques of HPLC.

Among the agents which attack specific groups along GAG chains are the Eliminase class of enzymes which cleave at uronic acid residues and which include, for example, heparinase (EC 4.2.2.7), chondroitinase ABC (EC 4.2.2.4), chondroitinase AC (EC 4.2.2.5), and chitinase (EC 3.2.1.14). Alternatively, a mixture may be treated with nitrous acid (HONO), which attacks N-acetyl-D-glucosamine monosaccharide units, resulting in chain cleavage. In each case, the average sizes and number of GAG species generated after treatment may be modulated by varying the

concentration of agent used and time of agent exposure. In general, the lower limit for a GAG molecule of the invention will be one consisting of 2-3 monosaccharide units. While there is no strict upper limit for the number of constituent monosaccharide units, the preferred upper limit will be 10-15 monosaccharide units per GAG molecule, and will most preferably consist of 6-8 monosaccharide units per molecule.

The heterogeneity of the mixture may be further reduced by separating elements of the mixture according to molecular weight. Separation may be accomplished by gel filtration and/or ion exchange chromatography. Each molecular weight class thus separated consists of a group of oligomers (e.g., of 2, 4, 6, 8, etc. monosaccharides depending on the molecular weight class). It is important to note that, because each of the agents described above attacks different groups along the GAG chain, the composition of molecules in a single molecular weight class will differ according to which treatment was administered prior to molecular weight separation.

The individual components of a single molecular weight class may be purified to homogeneity using high performance liquid chromatography (HPLC) techniques, preferably ion exchange chromatography, which are well known to those of skill in the art.

The purified ECM components of the invention described in this section may be modified using a variety of techniques which are well known to those of ordinary skill in the art. Such techniques modify GAGs at specific groups along a GAG chain, and may be applied before and/or after separation according to molecular weight and/or HPLC purification. For example, GAGs may be further sulfated by the addition

of sulfate groups to amino ($-NH_2$) and/or hydroxyl groups (-OH) along the polysaccharide chain. Sulfation may be achieved via addition sulfur trioxide in pyridine. Further, GAG chain hydroxyl groups may be acylated and/or carboxyl groups ($-CO_2H$) may be esterified ($-CO_2S$, where "S" may be an aryl, alkyl, alkenyl, or alkynyl moiety). Acylation of hydroxyl groups made be achieved by treatment with acyl chlorides in pyridine. Esterification of carboxyl groups may be performed by activation with dicyclohexyl carbodiimide in an alcohol. The identity of the alcohol is dependent upon the identity of the desired resulting ester(s). Additionally, acylated groups may be deacylated, or amino groups may be aminated (-NHT, where "T" may be an aryl, alkyl, alkenyl, or alkynyl moiety. Deacylation may accomplished with the addition of lithium in water. The amidation of amine groups may be accomplished by treatment with acyl chlorides in pyridine. Further, in the case of components that have been Eliminase treated, nucleophilic modification of the Eliminase product(s) may be performed by Michael addition of thiols (i.e., thiol and lithium hydroxide).

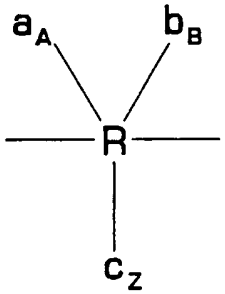
4.2 GAG MIMICS

In addition to purified ECM components and their derivatives, the well-defined compounds of the invention may also include GAG mimics. GAGs play an essential role in conferring onto cartilaginous tissue its unique material properties such as durability, stiffness, resiliency, and viscoelasticity. GAG mimics, therefore, refer here to molecules possessing the ability to substitute or partially substitute, for the presence of GAG molecules in conferring onto cartilage at least some of its essential properties,

which include, but are not limited to, those described above.

Among the molecules that may be used as GAG mimics are sulfated homopolymers or heteropolymers ranging from about 2 to about 15 monomeric units per molecule, with molecules ranging from about 6 to about 10 monomeric units being preferred. Each of the monomeric units should be sulfated or sulfatable, i.e., should contain 1) at least one amino ($-NH_2$) or hydroxyl (-OH) group to which a sulfate group may be attached, or 2) at least one $-SO_4^-$ or $-NSO_3^-$ group. The ratio of sulfates to monosaccharide units should, on average, be at least 0.5 (i.e., at least 1 sulfate group for every 2 monosaccharide units). In addition, each monomeric unit may contain a carboxyl (-COOH), carboxylate ($-COO^-$) or carboxoate (-COOU, where "U" may be an alkyl, alkenyl, alkynyl, or aryl moiety) group. The GAG mimic monomer may further contain aminate groups (-NHT, where "T" may be an alkyl, alkenyl, alkynyl, or aryl moiety), and or acyl groups (-COV, where "V" may be an alkyl, alkenyl, alkynyl, or aryl moiety).

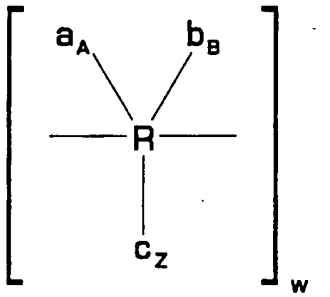
A GAG mimic monomeric unit of the type provided for herein can, therefore, be represented as in (I), solely for the purposes of illustration and not by way of limitation:



(I)

where R can include, but is not limited to, any simple monomeric unit or polymerizable unit including, but not limited to a hexuronic acid (Hex A; i.e. D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) a galactose monosaccharide, hexosamine (i.e., D-glucosamine (GlcN) or D-galactosamine (GalN); "a" can include, but is not limited to -OH, $-SO_4^-$, or -COV (where "V" may be an aryl, alkyl, alkenyl, or alkynyl moiety; "b" can include, but is not limited to, $-NH_2$, $-NSO_3^-$, or -NHT (where "T" may be an alkyl, alkenyl, alkynyl, or aryl moiety); "c" can include, but is not limited to -COOH, $-COO^-$, or -COOU (where "U" may be an alkyl, alkenyl, alkynyl, or aryl moiety); "A", "B" and "Z" are integers ranging from 0 to 30, and the sum of A and B is greater than or equal to 1.

A GAG mimic of the type provided herein can be represented as in (II), solely for the purposes of illustration and not by way of limitation:



(II)

where R, a, b, c, A, B, and Z are as described in (I), and W, which represents the total number of monomeric units within a GAG mimic, is an integer ranging from about 2 to about 15, with 6 to 10 being preferred. Each monomeric unit(s) is(are) covalently linked to its adjacent monomeric unit(s). Additionally, the ratio of the sum of all sulfations per GAG mimic, (i.e., the sum of all A wherein a_A equals $-SO_4^-$ plus the sum of all B wherein b or b_B equals $-NSO_3^-$) to w must be

greater than or equal to 0.5. It is important to note that, because the GAG mimics of the invention may consist of heteropolymers as well as homopolymers, each monomeric unit, within the GAG mimic may differ from all other monomeric units within the mimic. That is, the monomer constituents (R, a_A, b_B, c_z) need not be identical with respect to each monomeric unit.

Among the groups of molecules that may act as GAG mimics are sulfated homopolymers or heteropolymers of aromatic compounds, which include, but are not limited to aromatic dyes such as Congo Red and Suramin. Known aromatic compounds within this group of potential GAG mimics may be used, or, alternatively, unique aromatic polymers may be synthesized. Synthesis techniques by which such polymers may be made include standard solid phase or solution condensation or polymerization chemistry. Among other groups of molecules that may act as GAG mimics are sulfated polysaccharide-like molecules such as Suramin or Suramin analogs. Known compounds within this group of potential GAG mimics may be used, or unique polysaccharide-like molecules may be obtained as, for example, degradation products of larger polysaccharides. Such degradation procedures may include standard enzymatic (for example, Eliminase cleavage) and/or chemical (for example, nitrous acid treatment) procedures.

Additionally, molecules such as those described here may be modified according to techniques similar to those described in Section 4.1, above. Briefly, such modifications may include, but are not limited to sulfation, acylation, deacylation, esterification, amination, and nucleophilic modification. For example, GAG mimics may be further sulfated by the addition of sulfate groups to amino ($-NH_2$) and/or hydroxyl groups (-OH) along the GAG mimic chain.

Further, GAG mimic chain hydroxyl groups may be acylated and/or carboxyl groups ($-CO_2H$) may be esterified ($-CO_2S$, where "S" may be an alkyl, alkenyl, alkynyl, or aryl moiety). Additionally, acylated groups may be deacylated, or amino groups may be aminated (-NHT, where "T" may be an alkyl, alkenyl, alkynyl, or aryl moiety). Further, in the case of components that have been Eliminase treated, nucleophilic modification of the Eliminase product(s) may be performed by Michael addition of thiols.

4.3 BIOACTIVE MOLECULES

The purified compounds of the invention may be used alone or in conjunction with bioactive molecules for the repair of damaged cartilage such as is seen in OA. Such bioactive molecules may include, but are not limited to growth factors and/or metalloproteinase inhibitors.

Growth factors that may be used in conjunction with the purified compounds of the invention include, but are not limited to cartilage-derived growth factors, members of the TGF-β growth factor superfamily, connective tissue activating peptides, platelet-derived growth factor, fibroblast growth factor, and insulin and insulin-like growth factors I and II. Metalloproteinase inhibitors that may be used in conjunction with the purified compounds of the invention include, but are not limited to tissue inhibitors of metalloproteinases (TIMP1 and TIMP2) and plasminogen activator inhibitor (PAI-1).

4.4 SCREENING METHODS

Molecules which include, but are not limited to those of the type described in Sections 4.1, and 4.2 above, may be screened, using a variety of techniques,

for their ability to repair damaged cartilage. For example, candidate cartilage repair molecules may be initially selected for their ability to bind bioactive molecules known to influence cartilage metabolism. In addition, candidate cartilage repair molecules may be tested for their effects on chondrocyte cell cultures and/or OA animal models. These molecules may be tested alone, and in conjunction with bioactive molecules which include, but are not limited to the types described above, in Section 4.3.

Potential candidate cartilage repair molecules may be initially selected via their ability to bind bioactive molecules known to influence cartilage metabolism. The bioactive molecules that may be used in such a screening technique include, but are not limited to, those described, above, in Section 4.3. Briefly, such a screening technique may consist, first, of incubation of test compounds, individually or in a mixture, in the presence of bioactive molecules known to influence cartilage metabolism. Second, compounds not bound to the bioactive molecules are separated from the compound:bioactive molecule complexes, and, finally, the bound compound is separated from the bioactive molecule and recovered.

The bioactive molecules of this screening technique may be attached to a solid matrix, including, but not limited to agarose or plastic beads, microtiter wells, or petri dishes. After incubation, those compounds with no appreciable affinity for the bioactive molecule do not become attached to the solid matrix and may, therefore, be removed by rinsing of the matrix. Following removal of unbound compounds, the bound compounds may be eluted away from the bioactive molecules and recover d. Such bound compounds may be eluted using

standard techniques which include, but are not limited to an alteration of ionic strength, alteration of pH, and/or by the addition of chaotropic eluants.

Alternatively, the compounds and bioactive molecules may both exist free in solution during the incubation process. After incubation, the bioactive molecules may be removed from solution using, for example, standard immunoprecipitation techniques. Because any compound bound to the bioactive molecule would also be removed from solution, those compounds with an appreciable affinity for the bioactive molecules in use may be selected.

When single compounds are tested by this screening technique, their binding affinity for the bioactive molecule being used is determined. When mixtures of compounds are tested by this screening technique, however, not only is a molecule's affinity for the bioactive molecule identified, but, in addition, the screening technique serves as a partial purification scheme for potential cartilage repair molecules. Further purification of the selected compounds may be accomplished by repeating the screening procedure and/or utilizing separation and purification techniques such as those described, above, in Section 4.1. Therefore, large numbers of compounds may be quickly screened for their biomolecule binding affinity and, thus, for their potential as possible cartilage repair compounds. The compounds selected using this technique may then be tested for their biological effects on chondrocytes in vitro and/or in vivo.

Candidate cartilage repair molecules may also be screened using human chondrocyte cell culture systems. Human chondrocytes, either normal or OA, may be isolated directly from sterile cartilage specimens

which have been obtained from the hip or knee joint at the time of surgery or autopsy. The chondrocytes may be isolated from the specimens using techniques which are well known to those of ordinary skill in the art, and which may include treatment of the specimens by successive digestions with hyaluronidase, trypsin, and collagenase as described in Schwartz, E.R. (Cartilage Cells and Organ Culture, in Skeletal Research: An Experimental Approach, Simmons, D.J. and Kunin, A.S., eds., Academic Press: Orlando, FL, 1979) which is incorporated herein by reference in its entirety. Purified chondrocytes may be suspended in Ham's F12 medium (Ham, R.G., 1965, Proc. Natl. Acad. Sci. USA 53:288; Ham, R.G. and Murray, L.W., 1967, S. Cell Physiol. 70:275; Pechl, D.M. and Ham, R.G., 1980, In Vitro 16:526) supplemented with 12% fetal calf serum and antibiotics (e.g., penicillin 100 U/ml and streptomycin (100μg/ml), then the cells may be grown to confluency in 75-cm^2 flasks. Cells in first passage are used for the screening procedure.

For screening, cell culture media is supplemented with varying concentrations of test compounds, i.e., candidate cartilage repair molecules for varying lengths of time. In addition, the cell culture media may also be supplemented with varying concentrations of bioactive molecules in conjunction with the candidate cartilage repair molecules. Both normal and OA chondrocyte cell cultures will be treated with candidate cartilage repair molecules. After treatment, qualitative and quantitative effects on the cultures, including but not limited to growth rates, ECM composition, and concentrations of various metalloproteinases, growth factors, growth factor receptors, inhibitors and activators of cell division and/or differentiation and other cellular factors can

be measured. These measurements are also made on untreated OA cell cultures as well as treated and untreated normal cell cultures. Comparisons are made between the measurements obtained each of these types of cell samples, and are used to score the potential effectiveness of each cartilage repair candidate.

Chondrocyte growth rates may be measured using a DNA synthesis assay. For such an assay, newly synthesized DNA may be labeled. For example, ^{3}H-thymidine (approximately 10μCi/ml) may be added to the cell culture media for a short period of time (1 hour, for example). Other labels may be utilized as well, including but not limited to biotinylated thymidine, fluorescently-labeled thymidine, or digoxigenin-labeled thymidine. After labeling, cells may be harvested, rinsed with phosphate buffered saline (PBS) and cold 0.3M perchloric acid ($HClO_4$) to extract unincorporated thymidine pools, and the amount of label incorporated into newly synthesized DNA may be measured using techniques well known to those of skill in the art. For example, DNA labeled with ^{3}H-thymidine may be obtained by determining the level of ^{3}H-radioactivity present in harvested cells' DNA.

Changes in a culture's ECM composition may be assayed in a variety of ways. For example, a determination of the total amount of collagen present may be made. In one such technique, utilized by Peterkofsky, B. and Diegelmann, R. (1971, Biochemistry 10:988-994) which is incorporated herein by reference in its entirety, cells are incubated in fresh media for 24 hours prior to being placed in serum-free media containing B-aminoproprionitrile (BAPN), a collagen crosslinking inhibitor, and ^{3}H-proline (2.5μCi/ml). Total collagen amounts may then be estimated in the culture medium and the cell layer as collagenase

sensitive material. Such measurements are taken over various time periods.

In addition to a total collagen analysis, an analysis of collagen chains may be performed. Utilizing one such procedure, chondrocyte cells may be labeled for 24 hours with 1.5 μCi/ml of ^{3}H-proline in serum-free media, at which time the cells are rinsed with PBS and disintegrated using ultrasonication. Samples are then dialyzed against 1mM ammonium bicarbonate (pH 7.5) for 72 hours and lyophilized. Samples may then be analyzed by one- or two-dimensional polyacrylamide gel electrophoresis, and the labeled collagens may be identified by fluorography, using standard techniques well known to those of ordinary skill in the art.

Additionally, proteoglycan synthesis may be measured using a number of procedures. Using one such procedure, a chondrocyte cell culture may be labeled for 24 hours. This label may include, but is not limited to ^{35}S-sulfate (at a concentration of approximately 10μCi/ml) or C-^{3}H-glucosamine (at a concentration of approximately 2.5μCi/ml). The rate of proteoglycan synthesis may be determined by then measuring the ^{35}S or ^{3}H incorporation into the GAG chains. The radioactive GAGs present in the medium and cell layer may be extracted following pronase (type XIV, Sigma Chemical Co.) digestion, in the presence of a Tris-HCl 0.1 M buffer, pH 7.5, containing 0.005 M calcium chloride, for 48 hours at 50°C. GAGs are then purified by alternating precipitations with cetylpyridinium chloride and ethanol as described in Jouis, V. et al. (FEBS Lett. 186:233-240, 1985), which is incorporated by reference herein in its entirety. GAG radioactivity may then be measured using, for example, liquid scintillation

counting methods which are well known to those of ordinary skill in the art.

Alternatively, proteoglycan synthesis may be measured by incubating chondrocytes in media containing [D-1-^{14}C] glucosamine hydrochloride (Amersham, Arlington Heights, IL) at a concentration of approximately 2 μCi/ml. Cells are incubated for 24 hours, then rinsed with PBS and subsequently incubated for 1 hour in PBS containing 5g/L glucosamine, rinsed with PBS, and ultrasonicated. Radioactivity that has been incorporated into GAGs may then be counted using, for example, a B-Counter.

Proteoglycan amounts may be measured by digesting chondrocytes according to the method of Oegema et al., (Oegema, T.R. et al., 1981, J. Biol. Chem. 256:1015-1022), which is incorporated herein by reference in its entirety. Digestion may be performed for 18 hours at 56°C, after which time samples may be cooled and then frozen at -20°C until being assayed. Proteoglycan levels may be measured using a metachromatic dye such as 1, 9-dimethylene blue (Farndale et al., 1982, Connect. Tiss. Res. 9:247-248) and comparing the intensity of staining to a GAG or proteoglycan standard, such as a commercial preparation of chondroitin sulfate (Sigma Chemical Co.).

Gross observations of proteoglycan-containing cartilage ECM within chondrocyte cultures may also be made. For example, chondrocytes may be stained, and proteoglycan-containing ECM material may be visualized, with a dye including, but not limited to alcian blue (pH 2.8; Sigma Chemical Co.).

The levels of various cellular factors within treated chondrocyte cultures may also be measured using, for example, standard ELISA immunoassay

techniques which are well known to those of ordinary skill in the art. The factors whose levels may be measured include, but are not limited to tissue inhibitors of metalloproteinases (TIMP1 and TIMP2), plasminogen activator inhibitor (PAI-1), tissue plasminogen activator (TPA), interleukin 1 (IL-1), and tumor necrosis factor (TNF).

Candidate cartilage repair compounds, alone and/or in conjunction with bioactive molecules, may be tested for their ability to alleviate or prevent the onset of OA in animal models. Normal and OA animals may be treated with candidate cartilage repair compounds. The effects of treatment may be measured and compared to measurements derived from untreated normal and OA animals. Comparison of these measurements allows scoring of the effectiveness of the candidate compound in cartilage repair. Among the observations and measurements that may be performed on animals after treatment are macroscopic and microscopic evaluations of cartilage tissue and measurements quanitating the synthesis of ECM components, such as collagen and/or proteoglycans.

To more accurately model OA and the catabolic action of chondrocytes on cartilage ECM, chondrocyte cultures may be exposed to, for example, varying concentrations of interleukin-1-beta or retinoic acid. The conditions for the latter may be found in Morales and Roberts (Morales, T.I. and Roberts, A.B., 1992, Arch. Biochem. Biophys. 293:79-84), which is incorporated herein by reference in its entirety. Potential cartilage repair compounds and formulations may be evaluated in control and catabolically activated chondrocyte cultures to quantitate the protective efficacy of these agents.

Animal models may include, but are not limited to the Pond-Nuki model of canine OA, as described in Pond, M.J. and Nuki, G. (Ann. Rheum. Dis 32:387-388, 1973), which is incorporated herein by reference in its entirety. In this model, OA is experimentally induced by producing joint laxity through the sectioning of the anterior or cruciate ligament of the knee joint of the hind limb of an adult dog.

Once a potentially effective compound is found, the option exists to further increase the compound's ability for cartilage repair. The compound may be modified according to techniques such as those described above, in Sections 4.1 and 4.2, and the modified compound(s) generated may subsequently be tested for its effectiveness in cartilage repair, alone and/or in conjunction with bioactive molecules of the type, for example, are described above, in Section 4.3. Such a "selection-modification-selection" scheme may be utilized until a compound or compounds of the desired cartilage repair activity is obtained.

4.5 USES AND ADMINISTRATION OF FORMULATIONS CONTAINING PURIFIED CANDIDATE CARTILAGE REPAIR COMPOUNDS

The individual, well defined components of the invention, alone or in conjunction with bioactive molecules, may be employed to repair damaged hard tissue, especially damaged cartilage tissue as can be observed in OA. An effective amount of the well defined components of the invention would be introduced into a host such that they would contact the damaged tissue for a period of time sufficient to repair the damaged tissue. The components of the inv ntion may serve, to alleviate and possibly prevent

a prevalent human disease. Because, in addition to being a prevalent medical disease of humans, OA represents a serious veterinary problem, especially in the race horse and companion dog segments, the components of the invention may also be useful in the treatment of OA in these animals as well as in humans. The components of the invention may exert their effect by modulating elements of cartilage tissue metabolism. Such elements include, but are not limited to, stimulation of chondrocyte cell division, control of chondrocyte collagen and/or proteoglycan secretion, generation of a favorable proteinase to proteinase inhibitor ratio within the tissue of interest, and regulation of the production of cellular factors such as growth factors.

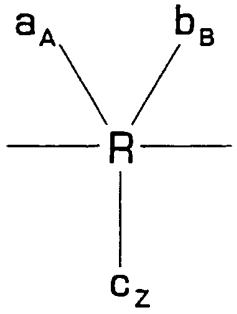
The components of the invention, alone and in conjunction with bioactive molecules, may be prepared as injectable formulations. For such injections, the agents of the invention may be formulated into aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological saline buffer. Injections may be local, with intraarticular administration into, or intramuscular administration adjacent to the diseased joint(s) of the OA individual.

It is apparent that many modifications and variations of this invention as set forth hereinabove may be made without departing from the spirit and scope thereof, and the invention is limited only by the terms of the appended claims.

CLAIMS:

What is claimed is:

1. A glycosaminoglycan mimic monomeric unit having the formula



where

R is a polymerizable unit;

"a" is -OH, $-SO_4^-$, or -COV,

where V is an alkyl, an alkenyl, an alkynyl, or an aryl moiety;

"b" is $-NH_2$, $-NSO_3^-$, or -NHT

where T is an alkyl, an alkenyl, an alkynyl, or an aryl moiety;

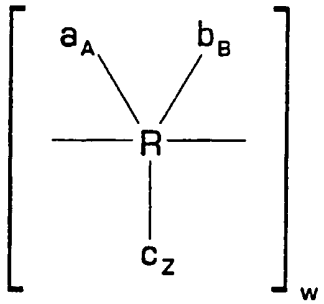
"c" is -COOH, $-COO^-$, or -COOU

where U is an alkyl, an alkenyl, an alkynyl, or an aryl moiety;

A, B and Z are integers ranging from 0 to about 30; and

the sum of A and B is greater than or equal to 1.

2. A glycosaminoglycan mimic having the formula



where R is a polymerizable unit;

"a" is -OH, $-SO_4^-$, or -COV,

where V is an alkyl, an alkenyl, an alkynyl, or an aryl moiety;

"b" is $-NH_2$, $-NSO_3^-$, or -NHT

where T is an alkyl, an alkenyl, an alkynyl, or an aryl moiety;

"c" is -COOH, $-COO^-$, or -COOU

where U is an alkyl, an alkenyl, an alkynyl, or an aryl moiety;

A, B, and Z are integers ranging from 0 to about 30;

W is an integer ranging from about 2 to about 15;

the sum of A and B is greater than or equal to 1; and

the ratio of the sum of all A wherein the "a" of a_A is $-SO_4^-$ and all B wherein the "b" of b_B is $-NSO_3^-$, to W is greater than or equal to 0.5.

3. The glycosaminoglycan mimic of Claim 2 wherein at least one R, "a", "b", or "c" is not identical to all other R, "a", "b", or "c", respectively.

4. The monomeric unit of Claim 1 or the glycosaminoglycan mimic of Claim 2 or 3 wherein R is a

galactose monosaccharide, a hexuronic acid, a hexosamine, or an aromatic moiety.

5. The monomeric unit or glycosaminoglycan mimic of Claim 4 wherein the hexuronic acid is D-glucuronic acid or L-iduronic acid.

6. The monomeric unit or glycosaminoglycan mimic of Claim 4 wherein the hexosamine is D-glucosamine or D-galactosamine.

7. The glycosaminoglycan mimic of Claim 2 or 3 wherein the glycosaminoglycan mimic is an aromatic dye.

8. The glycosaminoglycan mimic of Claim 7 wherein the aromatic dye is Congo Red or Suramin.

9. A purified extracellular matrix component, wherein the extracellular matrix component is a purified glycosaminoglycan species or a purified proteoglycan species.

10. The purified extracellular matrix component of Claim 9 wherein the purified extracellular matrix component is a purified glycosaminoglycan species each member of which comprises at least two covalently attached monosaccharide units, or the purified extracellular matrix component is a purified proteoglycan species each member of which comprises a core protein covalently attached to a glycosaminoglycan which comprises at least two covalently attached monosaccharide units.

11. The purified glycosaminoglycan or proteoglycan species of Claim 10 wherein each member of the purified glycosaminoglycan species has a molecular weight within 1000 daltons of the molecular weight of every other member of the glycosaminoglycan species, all members of the glycosaminoglycan species have a substantially identical polysaccharide composition, and all members of the glycosaminoglycan species have a substantially identical sulfation pattern, or each member of the purified proteoglycan species has a molecular weight within 1000 daltons of the molecular weight of every other member of the purified proteoglycan species, all members of the purified proteoglycan species have a substantially identical core protein identity, and all members of the purified proteoglycan species have a substantially identical glycosaminoglycan modification pattern.

12. The purified glycosaminoglycan or proteoglycan species of Claim 11 wherein each member of the purified glycosaminoglycan species comprises no more than about 15 covalently attached monosaccharide units, or each member of the purified proteoglycan species comprises no more than about 15 covalently attached glycosaminoglycan monosaccharide units.

13. The purified glycosaminoglycan or proteoglycan species of Claim 11 wherein each member of the purified glycosaminoglycan species comprises no more than about 8 covalently attached monosaccharide units, or each member of the purified proteoglycan species comprises no more than about 8 covalently attached glycosaminoglycan monosaccharide units.

14. The purified glycosaminoglycan or proteoglycan species of Claim 11 wherein at least one glycosaminoglycan or proteoglycan monosaccharide unit is a galactose monosaccharide, a hexuronic acid, or a hexosamine.

15. The purified glycosaminoglycan or proteoglycan species of Claim 14 wherein at least one hexuronic acid is D-glucuronic acid or L-iduronic acid.

16. The purified glycosaminoglycan or proteoglycan species of Claim 14 wherein at least one hexosamine is D-glucosamine or D-galactosamine.

17. The purified glycosaminoglycan or proteoglycan species of Claim 11 wherein the purified glycosaminoglycan or proteoglycan species is acetylated, esterified, acylated, aminated, carboxylated or sulfated.

18. The purified glycosaminoglycan or proteoglycan species of Claim 11 wherein the purified glycosaminoglycan or proteoglycan species is chondroitin-4-sulfate, chondroitin-6-sulfate, hyaluronic acid, heparin, heparin sulfate, keratan sulfate, dermatan sulfate, poly-N-acetyl-glucosamine, or poly-N-glucosamine.

19. A method for treating damaged hard tissue comprising contacting the damaged hard tissue with an effective amount of the glycosaminoglycan mimic of Claim 2 or 3 so that the damaged tissue is at least partially repaired.

20. A method for treating damaged hard tissue comprising contacting the damaged hard tissue with an effective amount of the purified glycosaminoglycan or proteoglycan species of Claim 11 so that the damaged tissue is at least partially repaired.

21. A method for the treatment of osteoarthritis comprising:

(a) comprising administering to an osteoarthritis-affected patient the glycosaminoglycan mimic of Claim 2 or 3; and

(b) contacting an osteoarthritis-diseased tissue of the patient with an effective amount of the glycosaminoglycan mimic so that the osteoarthritis is at least partially alleviated.

22. A method for the treatment of osteoarthritis comprising administering to an osteoarthritis-affected patient the purified glycosaminoglycan or proteoglycan species of Claim 11 so that the osteoarthritis is at least partially alleviated.

23. The method of Claim 19 or 20 wherein the hard tissue is cartilage.

24. The method of Claim 20 or 21 wherein the glycosaminoglycan mimic or purified glycosaminoglycan species is sulfated.

25. The method of Claim 20 or 21 wherein the glycosaminoglycan mimic or purified glycosaminoglycan species is chondroitin-4-sulfate, chondroitin-6-sulfate, hyaluronic acid, heparin, heparin sulfate, keratan sulfate, dermatan sulfate, poly-N-acetyl-glucosamine, or poly-N-glucosamine.

26. The method of Claim 19, 20, 21, or 22 further comprising administering an effective amount of a bioactive molecule in conjunction with the glycosaminoglycan mimic, or purified glycosaminoglycan or proteoglycan species.

27. The method of Claim 26 wherein the bioactive molecule is a growth factor or a metalloproteinase inhibitor.

28. The method of Claim 27 wherein the growth factor is a cartilage-derived growth factor, a member of the TGF-β growth factor superfamily, a connective tissue activating peptide, a platelet-derived growth factor, a fibroblast growth factor, an insulin growth factor, an insulin-like growth factor.

29. The method of Claim 27 wherein the metalloproteinase inhibitor is TIMP1, TIMP2, or plasminogen activator inhibitor.

30. The method of Claim 21 or 22 wherein the patient is a human, a dog or a horse.

31. A method for screening compounds for their ability to repair damaged cartilage comprising:

(a) incubating a test compound with a bioactive molecule known to influence cartilage metabolism;

(b) separating the test compounds which do not bind to the bioactive molecule; and

(c) recovering the compounds that bind to the bioactive molecule.

32. The method of Claim 31 wherein the bioactive molecule is attached to a solid matrix.

33. The method of Claim 32 wherein the solid matrix is agarose, a plastic bead, a microtiter well, or a petri dish.

34. A method for screening compounds for their ability to repair damaged cartilage comprising:

(a) incubating a chondrocyte cell culture in the presence of a test compound;

(b) measuring effects of the test compound on the chondrocyte cell culture.

35. The method of Claim 34 wherein the effect measured relates to chondrocyte cell culture growth rate, extracellular matrix composition, metalloproteinase concentration, growth factor concentration, growth factor recepter concentration, cell division inhibitor concentration, or cell division activator concentration.

36. The method of Claim 34 further comprising incubating the chondrocyte cell culture with a bioactive molecule in conjunction with the test compound.

37. The method of Claim 31 or 36 wherein the bioactive molecule is a growth factor or a metalloproteinase inhibitor.

38. The method of Claim 37 wherein the growth factor is a cartilage-derived growth factor, a member of the TGF-β growth factor superfamily, a connective tissue activating peptide, a platelet-derived growth factor, a fibroblast growth factor, an insulin growth factor, an insulin-like growth factor.

39. The method of Claim 37 wherein the metalloproteinase inhibitor is TIMP1, TIMP2, or plasminogen activator inhibitor.

40. A method for purifying an extracellular matrix component comprising:

(a) treating a heterogenous mixture of molecules with an agent that attacks specific groups along glycosaminoglycan chains so that the complexity of the mixture is reduced;

(b) separating the components of the mixture according to molecular weight; and

(c) obtaining an individual purified species or extracellular matrix component by separating the components of a molecular weight class.

41. The method of Claim 40 wherein the agent that attacks specific group along glycosaminoglycan chains is eliminase, heparinase, chondroitinase ABC, chondroitinase AC, chitinase, or nitrous acid.

42. The method of Claim 40 wherein separating the components of the mixture according to molecular weight is via gel filtration or ion exchange chromatography.

43. The method of Claim 40 wherein obtaining an individual purified species of extracellular matrix component by separating the components of a molecular weight class is accomplished via high performance liquid chromatography.

44. The method of Claim 40 wherein the purified extracellular matrix component is a purified glycosaminoglycan or proteoglycan species.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06490

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/54, 56, 62, 183, 613, 616, 617, 619, 646, 656, 657; 536/ 18.7, 21, 22, 33, 54, 55.1, 55.2, 115, 118, 119; 534/558, 561

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Abstracts, Volume 105, issued 1986, Nurgatin et al., "Acidic Hydrolysis of 2,3,5,6-Tetrasubstituted 1,4-Diformylpiperazines", see page 646, column 1, abstract number 78908h, Khim. Geterotsikl. Soedin., volume 9, issued 1985, pages 1265-1266, see the abstract.	1, 2
Y	AGENTS AND ACTIONS, Volume 40, No. 3-4, issued 1993, K. D. Brandt, "Compensation and Decompensation of Articular Cartilage in Osteoarthritis", pages 232-234, see the abstract.	1-8, 19-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
08 SEPTEMBER 1994

Date of mailing of the international search report
SEP 19 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
KATHLEEN KAHLER FONDA
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06490

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AMERICAN JOURNAL OF VETERINARY RESEARCH, Volume 51, No. 5, issued May 1990, M. J. Glade, "Polysulfated Glycosaminoglycan Accelerates Net Synthesis of Collagen and Glycosaminoglycans by Arthritic Equine Cartilage Tissues and Chondrocytes", pages 779-785, see the abstract.	1-8, 19-22
Y	ARTHRITIS AND RHEUMATISM, Volume 32, No. 6, issued June 1989, R. D. Altman et al., "Prophylactic Treatment of Canine Osteoarthritis with Glycosaminoglycan Polysulfuric Acid Ester", pages 759-766, see pages 759-760.	
Y	CLINICAL ORTHOPAEDICS AND RELATED RESEARCH, Volume 213, issued December 1986, D. S. Howell et al., "Articular Cartilage Breakdown in a Lapine Model of Osteoarthritis", pages 69-76, see the abstract.	1-8, 19-22
Y	CURRENT THERAPEUTIC RESEARCH, Volume 40, No. 6, issued December 1986, D. Burkhardt et al., "Laboratory Evaluation of Glycosaminoglycan Polysulphate Ester for Chondroprotective Activity: A Review", pages 1034-1053, see pages 1047-1049.	1-8, 19-22
Y	SCANDINAVIAN JOURNAL OF RHEUMATOLOGY, Volume 12, issued 1983, H. Vanharanta, "Glycosaminoglycan Polysulphate Treatment in Experimental Osteoarthritis in Rabbits", pages 225-230, see page 229.	1-8, 19-22
Y	US, A, 5,158,940 (LaROCCA ET AL.) 27 OCTOBER 1992, see column 2, lines 5-15.	1-8, 19-22
Y	US, A, 5,013,724 (PETITOU ET AL.) 07 MAY 1991, see column 21, line 64 to column 22, line 6.	1-8, 19-22
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 244, No. 1, issued 10 January 1969, S. W. Sajdera et al., "Proteinpolysaccharide Complex from Bovine Nasal Cartilage", pages 77-87.	
X	US, A, 5,036,056 (KLUDAS) 30 JULY 1991, see columns 5-12.	9-22 and 40-44
T, P	US, A, 5,246,013 (FRANK ET AL.) 21 SEPTEMBER 1993.	40-44
Y	US, A, 5,206,354 (SEDDON ET AL.) 27 April 1993, see column 3.	40-44

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06490

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,188,959 (HABERMAN) 23 FEBRUARY 1993, see column 19, line 61 to column 20, line 42.	40-44
Y	US, A, 5,171,674 (STEVENS ET AL.) 15 DECEMBER 1992, see column 28, lines 1-20.	40-44
Y	US, A, 5,071,436 (HUC ET AL.) 10 DECEMBER 1991, see column 1.	19-22 and 40-44
Y	US,A, 4,859,581 (NICOLSON ET AL.) 22 AUGUST 1989, see columns 1-4.	40-44

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 f first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 23-30
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-22 and 40-44

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06490

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 31/165, 31/075, 31/235, 31/215, 31/655, 31/715, 31/725, 31/73, 31/735; C07H 3/06, 7/033, 13/02; C08B 37/10; C07C 15/20, 15/24, 15/27, 211/43, 211/54, 225/02, 233/01, 245/02, 245/06

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/54, 56, 62, 183, 613, 616, 617, 619, 646, 656, 657; 536/ 18.7, 21, 22, 33, 54, 55.1, 55.2, 115, 118, 119; 534/558, 561

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN files searched: Registry, CA, WPIDS, Biosis, Embase. Search terms: osteoarthritis, arthritis, bone#, cartilage#, joint#, inflammation, glycosaminoglycan#, galactose, glucuronic, iduronic, hexosamine#, glucosamine#, galactosamine#, congo red, suramin, sulfated, sulfation, polysulf?, aminosulf, aminosulfon?, sulfamic, amidogen

APS file searched: USPAT. Search terms: ?cellular matrix, glycosaminoglycan#, proteoglycan#, extract?, purif?

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

I. Claims 1-22 and 40-44, drawn to compounds, methods of puirfication of said compounds, and methods of using said compounds for treatment of hard tissue damage and osteoarthritis.

II. Claims 31-39, drawn to methods of screening compounds for their ability to repair damaged cartilage.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features. The screeening methods of Group II do not require the compounds of Group I, but rather may be employed with any compound which is potentially active as a pharmaceutical to repair damaged cartilage.